

Study of the Cell-Transforming Ability of Amosite and Crocidolite Asbestos and the Ability to Induce Changes in the Metabolism and Macromolecular Binding of Benzo(a)pyrene in C3H10T $\frac{1}{2}$ Cells

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The cell transforming ability of asbestos dusts was investigated using C3H10T $\frac{1}{2}$ murine fibroblasts. In a series of experiments, crocidolite and amosite caused no increase in the number of transformed colonies over that seen in cultures from untreated cells. The dusts were, however, capable of augmenting the oncogenic effect of benzo(a)pyrene. This synergistic effect was evident when fibers and chemicals were added to cultures as simple mixtures and when benzo(a)pyrene was adsorbed to the surface of fibers. Asbestos dust did not, however, appear to exert its oncogenic enhancing effect by modifying the metabolism of benzo(a)pyrene in C3H10T $\frac{1}{2}$ cells.

Introduction

It is well established that exposure to amphibole asbestos leads to an increased incidence of lung cancer and mesotheliomata (1) but the mechanism by which it exerts its effect is largely unknown. On the basis of results obtained from chromosomal aberration studies (2) and the induction of point mutations (3) in Chinese hamster cells, it has been suggested that crocidolite may behave as a "conventional" carcinogen causing genetic damage. The apparent inactivity of asbestos dusts in bacterial mutation tests (4, 5) and negative results in sister chromatid exchange analysis (6) would, however, suggest that, at best, mineral fibers are only weakly genotoxic.

Epidemiological evidence linking asbestos exposure and cigarette smoking in the etiology of lung cancer (7-10) has provoked many experiments based on the supposition that these fibers might exert their biological effect by enhancing the tumorigenicity of some chemical carcinogens. These studies have recently been reviewed (11).

The aim of the work presented here was (a) to determine whether amphibole asbestos fibers possessed the ability to cause the *in vitro* transformation of C3H10T $\frac{1}{2}$, (b) to explore the possibility that they might enhance the cell-transforming activity of the polycyclic aromatic hydrocarbon benzo(a)pyrene (BaP) and (c) to ascertain whether they can cause any change in the ability of C3H10T $\frac{1}{2}$ cells to metabolize BaP.

Materials and Methods

Asbestos

The UICC standard samples of crocidolite and amosite (12) were used. Milled amosite was prepared as reported previously (13) and was milled for 4 hr.

Chemicals

Benzo(a)pyrene (BaP) was obtained from the Sigma Chemical Co., Poole, Dorset, UK. Fetal calf serum and other tissue culture media and reagents were obtained from Flow Laboratories, Irvine, Scotland. [3 H]-Benzo(a)pyrene (20 mCi/mmol) was ob-

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tained from the Radiochemical Centre, Amersham, England. Both [^3H]-labeled and nonradioactive BaP were purified before use by eluting through a Sep Pak silica gel cartridge with petroleum ether. BaP was added to cultures in one of two ways: as a solution in acetone final concentration in medium (<0.5%) or adsorbed to the surface of the dust. This adsorption was carried out by suspending the dust in ethanol, adding the appropriate concentration of BaP solution (in acetone), and drying down at 80°C in a stream of nitrogen.

Dusts were sterilized by autoclaving (15 psi for 15 min) and then suspended in complete medium and added to the cultures to give the desired concentrations.

All solutions were prepared immediately prior to use.

Plastic Ware

Corning disposable Petri dishes were obtained from Kernicks Ltd., Pentwyn, Cardiff. Tissue culture flasks were from either Nunc (Gibco Europe Ltd., Paisley, Scotland) or Falcon (Becton Dickinson (UK) Ltd., Wembely, Mddx.). Other sources were found to be unsatisfactory.

Cell Culture

The 10T $\frac{1}{2}$ Cl 8 cell line derived from C3H mouse embryo fibroblasts (14) was received in this laboratory at passage 9 as the kind gift of Dr. W. J. Harris, Inveresk International Ltd. All experiments were carried out on cells from passage 10-13.

The cells were grown in Dulbecco's modification of Eagle's Medium with a concentration of sodium bicarbonate of 3.6 g/L to permit equilibration with a gas phase of 8% CO $_2$ in air. It was supplemented with twice the normal concentration of glutamine, plus heat-inactivated fetal calf serum (10% v/v) and contained penicillin (100 units/mL) and streptomycin (100 $\mu\text{g/mL}$).

Transformation Studies

Two methods based on the basic protocol of Reznikoff et al. (15) were used for transformation assays.

Studies in 6 cm Petri Dishes. Cells were seeded together with the agent under tests such that approximately 1000 colony forming units could be expected to survive on each plate. The cells were incubated at 37°C in an atmosphere of 8% CO $_2$ in air in the presence of the test agent. The medium was changed 48 hr later and thereafter twice weekly for 3 weeks, at which time the serum concentration was reduced to 5%. This medium was then changed weekly for 3 weeks more. The plates were fixed in formalin (10%), stained in aqueous methylene blue (1%) and, after washing and drying, the number of

type III transformed foci were counted by using the criteria described by Reznikoff et al. (15). Toxicity was assessed in this method by plating 400 cells into each of a series of 9 cm Petri dishes together with a range of concentrations of the agent under study. After 10 days these survival plates were fixed in formalin (10%), stained in methylene blue (1%), and the colonies counted. The toxicity of the agent at the concentration used in the transformation assay was estimated from the resulting survival curve.

Studies in 25 cm 2 Tissue Culture Flasks. Samples of C3H10T $\frac{1}{2}$ cells (5 mL, 200 cells/mL) from subconfluent cultures of C3H10T $\frac{1}{2}$ cells (passage 10) were distributed among 25 cm 2 tissue culture flasks (Nunc) which were placed in a 37°C humidified incubator with an atmosphere of 5% CO $_2$ in air. The flasks were incubated overnight with caps screwed on lightly to allow for equilibration of gas. At 24 hr after plating the cultures were treated with the test agents or positive control chemical dissolved in acetone (final concentration 0.5%).

The cultures were exposed to the chemical for 48 hr at 37°C, after which time a medium change was made. The culture medium was then changed twice weekly until the cells reached confluence; thereafter medium changes were once weekly. After 6 weeks the cultures were fixed, stained and scored for transformed foci as described by Reznikoff et al. (15).

Metabolism Studies

Purified [^3H]-labeled and unlabeled benzo(a)pyrene in acetone were added to confluent monolayers of treated or untreated of C3H10T $\frac{1}{2}$ cells (Table 3) growing in 175 cm 2 tissue culture flasks to give final concentrations of 10 $\mu\text{Ci/mL}$, 0.5 $\mu\text{g/mL}$. After a 24 hr incubation at 37°C in an atmosphere of 8% CO $_2$ in air, the medium and cells were separated and stored at -60°C under an atmosphere of nitrogen until analyzed.

The medium was extracted three times with ether (2 vol), and aliquots were examined for organic-soluble metabolites by thin-layer chromatography TLC in benzene: petroleum ether (1:1). The organic-soluble metabolites were quantified following cutting of the chromatogram and liquid scintillation counting in a Intertechnique SL 4000. The radioactivity in the water-soluble conjugate metabolites remaining in the extracted medium was determined by liquid scintillation counting.

Estimation of Radioactivity Bound to Cellular Material

Cellular macromolecules were precipitated and extracted by using published methods (16).

Results

Transformation Assays

Amosite and crocidolite were toxic to 10T $\frac{1}{2}$ cells at similar or lower concentrations to those reported for other cell lines (17). The results of the cytotoxicity studies showed that a coincident exposure of 10T $\frac{1}{2}$ cells to dust and BaP resulted in greater cell death than when cells were treated with either of the agents alone. This increase in toxicity following coincident exposure, although variable, suggested that the effect was synergistic rather than cumulative (Fig. 1 and Table 1). The cytotoxicity of BaP adsorbed to the dust, although greater than that of the separate toxicities, was, however, less than that of the simple mixture (Fig. 1). The milled amosite sample was less toxic than the UICC sample, which is in agreement with results reported with V79-4 cells (13).

The results of the four transformation assays are shown in Tables 1 and 2, experiments 1-3 having been performed with 6 cm diameter tissue culture Petri dishes and experiment 4 with 25 cm² flasks. Experiments 1-3 were carried out with different numbers of cells added to each treatment group in an attempt to keep the numbers of surviving cells constant. This proved difficult in practice, since survival varied from experiment to experiment, so that

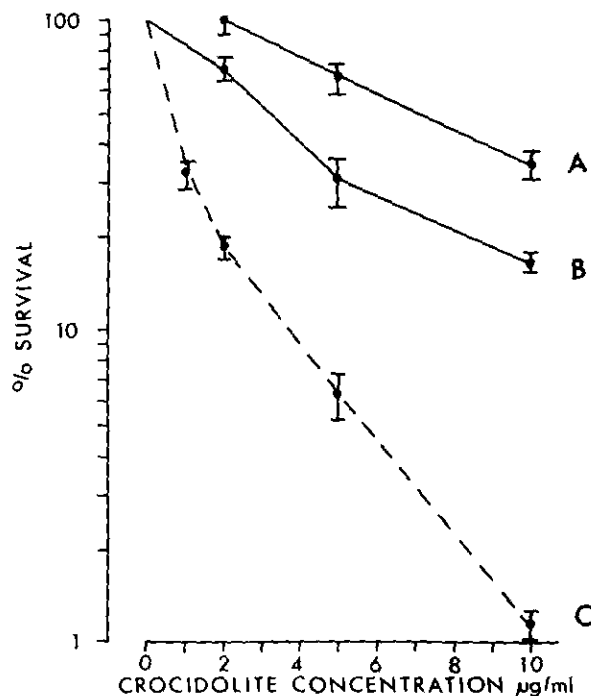


FIGURE 1. Survival curves for C3H10T $\frac{1}{2}$ cells treated with (A) UICC crocidolite, (B) crocidolite with adsorbed benzo(a)pyrene and (C) crocidolite and benzo(a)pyrene in acetone solution. The ratio of BaP to crocidolite was kept constant at 0.06 μ g BaP/ μ g crocidolite. The mean survival and 95% confidence limits are shown.

Table 1. Survival of treated cultures as a fraction of untreated controls and as surviving number of cells per culture and calculated transformation frequencies.

Expt.	Treatment	Survival, %	Surviving cells/culture	Transformation frequency per 10 ⁵ cells (95% confidence limits)
Expt. 1 ^a	Control	100	472	0(-116)
	Amosite, 5 μ g/mL	26	349	0(-157)
	Crocidolite, 5 μ g/mL	69	662	0(-121)
	Milled amosite, 5 μ g/mL	100	960	0(-77)
	BaP, 0.3 μ g/mL	100	941	177(63-379)
Expt. 2 ^a	Control	100	563	142(24-439)
	Amosite, 5 μ g/mL	73	951	39(22-171)
	BaP, 0.3 μ g/mL	98	1273	157(68-304)
	Amosite, 5 μ g/mL, and BaP, 0.3 μ g/mL	16	701	648(373-1030)
	Amosite, 5 μ g/mL with BaP adsorbed to equivalent of 0.3 μ g/mL	37	1470	240(129-402)
Expt. 3 ^a	Control	100	480	47(27-200)
	Crocidolite, 5 μ g/mL	72	691	0(-58)
	BaP, 0.3 μ g/mL	64	604	113(28-293)
	Crocidolite, 5 μ g/mL, with BaP adsorbed to equivalent of 0.3 μ g/mL	19	457	912(568-1370)
Expt. 4 ^b	Control H ₂ O	100	1200	0(-80)
	Control acetone	100	1200	0(-80)
	Crocidolite, 10 μ g/mL	78	936	0(-11)
	BaP, 0.1 μ g/mL	100	1200	42(2-173)
	BaP, 2.5 μ g/mL	49	588	2980(2100-4070)
	Crocidolite, 10 μ g/mL, and BaP, 0.1 μ g/mL	29	348	684(245-1470)
	Crocidolite, 10 μ g/mL, with BaP adsorbed to equivalent of 0.1 μ g/mL	95	1140	526(282-883)

^aPetri dish culture.

^bFlash culture.

the alternative protocol with constant numbers of cells was used in experiment 4. Frequency distributions for each experiment showing the numbers of cultures with transformed colonies, actual numbers of type III foci/culture, cumulative numbers of foci and actual numbers of surviving cultures are shown in Table 2. These results showed that BaP at concentrations of 0.1 and 0.3 $\mu\text{g/mL}$ produced only very few transformed foci, while none of the dusts examined produced any significant increase in transformed cultures as compared to the negative controls. The cocubation of crocidolite or amosite and BaP with 10T $\frac{1}{2}$ cells, however, caused a significant increase in numbers of transformed foci, i.e., a 2.3-fold increase in experiment 4 over the number of transformed foci produced by BaP alone. BaP adsorbed onto dust also had a greater transforming ability than that of BaP or dust alone, producing 1.8, 2 and 6.1 times the number of foci in experiments 2, 3 and 4, respectively.

In addition to the observed increases in absolute numbers of transformed foci, there was also a parallel increase in transformation frequency (Table 1), the enhancement ratios being calculated as 4.1 and

16.3 times for the mixtures and 1.5, 8 and 12 times for adsorbed BaP.

Metabolism Studies

[^3H]-BaP was metabolized to organic solvent- and water-soluble products by both crocidolite-treated and untreated cultures of C3H10T $\frac{1}{2}$ cells (Table 3). There was no significant difference in the amounts of organic-soluble metabolites produced by treated and untreated cultures in any of the three separate experiments. A significant reduction in the proportion of [^3H]-BaP metabolized to water-soluble products was observed in cultures treated with the hydrocarbon adsorbed to the surface of the crocidolite (expt. 3). This alteration in the production of water-soluble metabolites was not, however, accompanied by changes in other measured parameters, i.e., protein- and DNA-bound metabolites.

The levels of [^3H]-BaP bound to cellular macromolecules of the various treatment groups are shown in Table 3. The results of the experiments were variable. In experiment 1 there was significantly higher binding of the [^3H]-BaP to the DNA of

Table 2. Distribution of type III transformed foci, total numbers of foci and mean number of foci per culture.

Expt.	Treatment	No. of cultures with <i>n</i> type III foci							No. dishes with foci/ total number of dishes	Mean number of foci per culture
		0	1	2	3	4	5	Other		
Expt. 1 ^a	Control	35							0/35	0
	Amosite, 5 $\mu\text{g/mL}$	35							0/35	0
	Crocidolite, 5 $\mu\text{g/mL}$	24							0/24	0
	Milled amosite, 5 $\mu\text{g/mL}$	26							0/26	0
	BaP, 0.3 $\mu\text{g/mL}$	25	5						5/30	0.17
Expt. 2 ^a	Control	23	2						2/25	0.08
	Amosite, 5 $\mu\text{g/mL}$	26	1						1/27	0.04
	BaP 0.3 $\mu\text{g/mL}$	29	5	1					6/35	0.20
	Amosite, 5 $\mu\text{g/mL}$, and BaP, 0.3 $\mu\text{g/mL}$	23	6	3	1				10/33	0.45
	Amosite, 5 $\mu\text{g/mL}$, with BaP adsorbed to equivalent of 0.3 $\mu\text{g/mL}$	26	6		2				8/34	0.35
Expt. 3 ^a	Control	43	1						1/44	0.02
	Crocidolite, 5 $\mu\text{g/mL}$	48							0/48	0
	BaP, 0.3 $\mu\text{g/mL}$	41	3						3/44	0.07
	Crocidolite, 5 $\mu\text{g/mL}$, with BaP adsorbed to equivalent of 0.3 $\mu\text{g/mL}$	39	3	4		1			8/47	0.43
Expt. 4 ^b	Control H $_2$ O	20							0/20	0
	Control acetone	20							0/20	0
	Crocidolite, 10 $\mu\text{g/mL}$	19							0/19	0
	BaP, 0.1 $\mu\text{g/mL}$	19	1						1/20	0.05
	BaP, 2.5 $\mu\text{g/mL}$	6	5	2	3	3	1		14/20	1.75
	Crocidolite, 10 $\mu\text{g/mL}$, and BaP 0.1 $\mu\text{g/mL}$	19		1	1				2/21	0.24
	Crocidolite, 10 $\mu\text{g/mL}$, with BaP adsorbed to equivalent of 0.1 $\mu\text{g/mL}$	19						1 with 12 foci	1/20	0.60

^aPetri dish culture.

^bFlask cultures.

the untreated cells compared to the crocidolite-treated cultures ($p > 0.05$). Experiment 2 showed a reverse of this effect, with significantly higher protein and DNA binding being observed in the crocidolite-treated cultures ($p > 0.05$). In the third experiment the only significant difference in macromolecular binding between treatment and control cultures was an increase in DNA binding ($p > 0.05$) in cells treated with [^3H]-BaP following a 24 hr exposure to crocidolite.

Discussion

The data presented here clearly show that crocidolite and amosite, at the concentrations tested, possessed no cell-transforming capability, but at similar doses were able to augment the oncogenic effect of benzo(a)pyrene. This effect was evident whether the agents were added as mixtures or with the BaP adsorbed to the surface of the fibers.

It would appear, therefore, that one effect of the coincident exposure of $10\text{T}^{1/2}$ cells to crocidolite and benzo(a)pyrene, at individually subeffective or at best slightly effective doses, is to cause a significant increase in the production of transformed foci. This *in vitro* synergistic effect is considered to parallel the *in vivo* situation where enhanced production of lung tumors is seen in asbestos workers who also smoke cigarettes (7).

These results are also consistent with the results of animal experiments in which the synergism of polycyclic aromatic hydrocarbons (PAH) and particulates for tumor production has been demonstrated (18-20).

As the way in which the neoplastic response of tissues and cells to BaP is augmented by asbestos is still to be established, a series of experiments was undertaken to discover if crocidolite exerted an effect by modifying the ability of C3H10T $^{1/2}$ cells to

metabolize polycyclic aromatic hydrocarbons (PAH). In the first of three experiments, the co-exposure of cells to asbestos (100 $\mu\text{g}/\text{mL}$) and [^3H]-BaP resulted in a reduction of the amount of BaP bound to DNA as compared to the nondusted controls. As the toxicity of crocidolite at 100 $\mu\text{g}/\text{mL}$ was very high, lower concentrations (10 $\mu\text{g}/\text{mL}$) were used in subsequent experiments. In these latter studies crocidolite treatment, either simultaneously with [^3H]-BaP or with a 24 hr pre-exposure of cells to the dust followed by addition of the isotope, resulted in an increase in the amount of [^3H]-BaP binding to DNA ($p > 0.05$). The enhanced binding of BaP to deoxynucleotide in cells exposed to asbestos 24 hr before PAH treatment has also been reported by other investigators (21).

In experiment 2, the exposure of cells in mixtures of [^3H]-BaP and crocidolite caused a reduction in the production of water-soluble metabolites. Although a repeat study (experiment 3) failed to confirm these findings, it was found in the same experiment that exposure of cultures to [^3H]-BaP bound to crocidolite fibers resulted in a significant reduction of water-soluble metabolites ($p > 0.05$).

The variability of the data generated by the metabolism experiments was such that no firm conclusions can be formulated as to whether or not asbestos dusts induced any significant, reproducible changes in BaP metabolism.

In conclusion, these data show that exposure to asbestos dusts alone resulted in no significant increase in transformed foci but that the dusts were capable of augmenting the oncogenic effect of benzo(a)pyrene. The results of the studies undertaken to investigate the possibility that asbestos may exert an effect through modifying the ability of cells to metabolize benzo(a)pyrene, although equivocal, do suggest this area should be the subject of future study.

Table 3. Pattern of metabolism of [^3H]-BaP by crocidolite-treated and untreated C3H10T $^{1/2}$ cells.

Expt.	Treatment	Water-soluble metabolites, nmole/mg protein	Organic-soluble metabolites, nmole/mg protein	Protein-bound metabolites, pmole/mg protein	DNA adducts, pmole/ μg DNA
1	BaP (0.5 $\mu\text{g}/\text{mL}$)	24.1 \pm 7.3	2.2 \pm 0.02	29.1 \pm 4.7	0.58 \pm 0.07
	BaP (0.5 $\mu\text{g}/\text{mL}$) + crocidolite (100 $\mu\text{g}/\text{mL}$)	19.05 \pm 3.7	2.16 \pm 0.46	22.8 \pm 0.45	0.38 \pm 0.02
2	BaP (0.5 $\mu\text{g}/\text{mL}$)	13.13 \pm 6.07	2.08 \pm 0.46	19.0 \pm 4.4	0.23 \pm 0.05
	BaP (0.5 $\mu\text{g}/\text{mL}$) + crocidolite (10 $\mu\text{g}/\text{mL}$)	4.6 \pm 1.0	1.97 \pm 0.83	32.3 \pm 0.6	0.39 \pm 0.01
3	BaP (0.5 $\mu\text{g}/\text{mL}$)	14.85 \pm 0.35	7.48 \pm 0.13	52.3 \pm 7.7	0.61 \pm 0.19
	BaP (0.5 $\mu\text{g}/\text{mL}$) + crocidolite (10 $\mu\text{g}/\text{mL}$)	16.9 \pm 0.7	8.7 \pm 0.79	61.4 \pm 1.0	0.79 \pm 0.22
	Crocidolite (10 $\mu\text{g}/\text{mL}$) with BaP adsorbed to equivalent of (0.5 $\mu\text{g}/\text{mL}$)	6.62 \pm 0.17	5.55 \pm 0.79	70.7 \pm 1.4	0.80 \pm 0.03
	Cultures treated with crocidolite (10 $\mu\text{g}/\text{mL}$) 24 hr prior to addition of BaP	16.88 \pm 0.53	7.86 \pm 0.96	75.6 \pm 1.3	1.56 \pm 0.16

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